The homologous regulators ANR of *Pseudomonas aeruginosa* and FNR of *Escherichia coli* have overlapping but distinct specificities for anaerobically inducible promoters

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The anaerobic transcriptional regulator ANR induces the arginine deiminase and denitrification pathways in *Pseudomonas aeruginosa* during oxygen limitation. The homologous activator FNR of *Escherichia coli*, when introduced into an *anr* mutant of *P. aeruginosa*, could functionally replace ANR for anaerobic growth on nitrate but not for anaerobic induction of arginine deiminase. In an FNR-positive *E. coli* strain, the ANR-dependent promoter of the *arcDABC* operon, which encodes the enzymes of the arginine deiminase pathway, was not expressed. To analyse systematically these distinct induction patterns, a *lacZ* promoter-probe, broad-host-range plasmid containing various –40 regions (the ANR/FNR recognition sequences) and –10 promoter sequences was constructed. These constructs were tested in *P. aeruginosa* and in *E. coli* expressing either ANR or FNR. In conjunction with the consensus –10 hexamer of *E. coli* σ^{70} RNA polymerase (TATAAT), the consensus FNR site (TTGAT....ATCAA) was recognized efficiently by ANR and FNR in both hosts. By contrast, when promoters contained the Arc box (TTGAC....ATCAG), which is found in the *arcDABC* promoter, or a symmetrical mutant FNR site (CTGAT....ATCAG), ANR was a more effective activator than was FNR. Conversely, an extended 22 bp, fully symmetrical FNR site allowed better activation with FNR than with ANR. Combination of the *arc* promoter –10 sequence (CCTAAT) with the Arc box or the consensus FNR site resulted in good ANR-dependent expression in *P. aeruginosa* but gave practically no expression in *E. coli*, suggesting that RNA polymerase of *P. aeruginosa* differs from the *E. coli* enzyme in –10 recognition specificity. In conclusion, ANR and FNR are able to activate the RNA polymerases of *P. aeruginosa* and *E. coli* when the –40 and –10 promoter elements are identical or close to the *E. coli* consensus sequences.

Keywords: *Pseudomonas aeruginosa*, anaerobic control, ANR, FNR, *arcDABC* operon

INTRODUCTION

The *anr* gene of *Pseudomonas aeruginosa* is required for the expression of physiological functions which are inducible under oxygen-limiting or anaerobic conditions: the arginine deiminase pathway, denitrification and cyanogenesis (Galimand *et al.*, 1991; Zimmermann *et al.*, 1991;

Sawers, 1991; Haas *et al.*, 1992; Arai *et al.*, 1995a; Ye *et al.*, 1995). The *anr* gene product is a protein which has 51 % amino acid sequence identity with the anaerobic transcriptional regulator FNR of *Escherichia coli* (Sawers, 1991; Zimmermann *et al.*, 1991). Several promoters of *P. aeruginosa* are positively controlled by ANR (Haas *et al.*, 1992; Arai *et al.*, 1995a); of these, the promoter of the *arcDABC* operon has been investigated in most detail (Galimand *et al.*, 1991; Gamper *et al.*, 1991). The *arc* operon encodes the enzymes of the arginine deiminase

Abbreviations: Ap, ampicillin (resistance); Cb, carbenicillin (resistance); Km, kanamycin (resistance); Tc, tetracycline (resistance).

pathway and thus enables *P. aeruginosa* to grow anaerobically with arginine as the sole energy source (Lüthi *et al.*, 1990). The -40 region of the *arc* promoter contains a sequence termed the Arc box, TTGAC....ATCAG, which is closely related to the consensus FNR-binding sequence TTGAT....ATCAA in *E. coli* (Spiro & Guest, 1990; Uden *et al.*, 1995). Mutational analysis of the *arc* promoter has shown that the Arc box is essential for ANR-dependent induction of the *arc* operon under oxygen-limiting conditions and that the -10 hexamer CCTAAT is also important for transcription of the *arc* genes. By contrast, the -35 region does not appear to be critical (Gamper *et al.*, 1991).

Transcriptional regulators of the FNR family occur in many Gram-negative and Gram-positive bacteria. Some members of this family, including FNR and ANR, are active under oxygen-limiting conditions; these regulators typically contain four essential and conserved cysteine residues, three of which lie in the N-terminal part of the protein (Green *et al.*, 1993; Spiro, 1994). It has been proposed that Fe²⁺ may be bound to the conserved cysteines in the active form of FNR and that oxidation of Fe²⁺ in the presence of O₂ may inactivate FNR (Uden *et al.*, 1995). However, the recent discovery of an iron-sulfur centre in FNR indicates that the mechanism of sensing the redox state may be more complex (Khoroshilova *et al.*, 1995). Some proteins of the FNR family, for instance DNR, do not have the conserved cysteine residues; DNR is a transcriptional regulator which is needed, in addition to ANR, for denitrification in *P. aeruginosa* (Arai *et al.*, 1995b).

A helix-turn-helix motif located in the C-terminal part of FNR is involved in the binding of the regulator to the conserved -40 region, as demonstrated by the effects of specific mutational changes in FNR (Spiro & Guest, 1987; Spiro *et al.*, 1990; Lazazzera *et al.*, 1993) and by footprinting and bandshift experiments (Green *et al.*, 1991; Sharrocks *et al.*, 1991; Green & Guest, 1994; Ziegelhoffer & Kiley, 1995). From these studies it is concluded that the FNR residues Glu-209, Ser-212 and Arg-213 in the second helix of the helix-turn-helix motif have a significant role in the recognition of the FNR box. The same residues are conserved in ANR (Zimmermann *et al.*, 1991). The FNR homologues of non-enteric bacteria all interact with -40 promoter regions having a TTGAT....ATCAA consensus (i.e. an FNR box) (Spiro, 1994). It has been verified experimentally that this consensus sequence is recognized by ANR (Lodge *et al.*, 1990; Galimand *et al.*, 1991).

The cloned intact *arcDABC* operon of *P. aeruginosa* is expressed at very low, uninduced levels in Fnr⁺ *E. coli* strains (Lüthi *et al.*, 1986; Galimand *et al.*, 1991). Strong *E. coli* promoters fused to the *arc* operon allow good expression of the arginine deiminase pathway enzymes in *E. coli*, indicating that the *arc* promoter rather than other features (such as codon usage) accounts for the poor expression of the *arc* operon in the heterologous host (Lüthi *et al.*, 1986; Gamper *et al.*, 1991). Low activity of the *arc* promoter in *E. coli* may be a consequence of poor

recognition of the Arc box by FNR and/or poor interaction between the *E. coli* RNA polymerase and the -10 hexamer CCTAAT of the *arc* promoter. Using synthetic, modular promoter constructs, we show here that both factors are important.

METHODS

Bacterial strains and growth conditions. The *P. aeruginosa* strains used were the wild-type PAO1 and its *anr* mutant derivative PAO6261 (Ye *et al.*, 1995). The construction of strain PAO6265, a PAO1 derivative carrying a *lacZ* transcriptional fusion in the chromosomal *arcD* gene, will be described elsewhere. The *E. coli* strains were MC1000 [$\Delta(lacIZYA)X74$ galU galK $\Delta(ara-leu) rpsL$; Eiglmeier *et al.*, 1989] and its mutant derivative JRG1728 [$\Delta(tyrR-fnr-trg)$; Spiro & Guest, 1987].

All incubations were carried out at 37 °C. *P. aeruginosa* was grown in yeast extract arginine medium under aerobic and oxygen-limiting conditions as described by Galimand *et al.* (1991). Anaerobic growth was tested on nutrient agar supplemented with 0.2% KNO₃ in an anaerobic jar. Plasmid maintenance was ensured by the addition of 100 µg carbenicillin ml⁻¹ or 125 µg tetracycline ml⁻¹. *E. coli* was grown in 60 ml Luria broth containing 0.5% glucose (Sambrook *et al.*, 1989), either aerobically with vigorous shaking in a baffled 500 ml flask or under oxygen limitation with gentle shaking in a hermetically sealed 125 ml bottle. Plasmid maintenance was ensured by the addition of 100 µg ampicillin ml⁻¹ or 50 µg kanamycin ml⁻¹. Both *P. aeruginosa* and *E. coli* cells were harvested in the early exponential phase ($< 5 \times 10^8$ cells ml⁻¹) for aerobic incubations and in early stationary phase (approximately 8×10^8 cells ml⁻¹) for oxygen-limiting conditions.

DNA manipulation and plasmid constructions. Plasmid DNA was isolated by the method of Del Sal *et al.* (1988) and sequenced by the chain-termination method with Sequenase (US Biochemical) according to the manufacturer's instructions. Plasmid pRK2501*fmr* (Tc, RK2 replicon, 11.7 kb), which carries the *E. coli fmr* gene on a 1.64 kb *Bam*HI-*Hind*III fragment (Shaw & Guest, 1982) in pRK2501 (Kahn *et al.*, 1979), was kindly supplied by S. Busby, University of Birmingham, UK. Plasmid pME3777 (Km, ColE1 replicon, 7.8 kb) carries the *P. aeruginosa anr* gene downstream of the *tac* promoter and is a Km-resistant derivative of pME3587 (Zimmermann *et al.*, 1991); a 1.2 kb Km cassette (the *Sma*I fragment of pUC4-KIXX; Pharmacia) was inserted into the *Sca*I site of pME3587. The modular plasmid pME3533 (Fig. 1) and its derivatives were constructed as follows. Three sets of complementary oligonucleotides ('modules') were synthesized: a 53-mer/61-mer pair with a 5' *Pst*I site and a 3' *Hind*III site carrying a fragment of the *E. coli lacZ* sequence (+1 transcriptional start site, ribosome-binding site and the first six codons of *lacZ*) (Dreyfus, 1988; Minton, 1984), two different 28-mer/20-mer pairs with a 5' *Sa*I site and a 3' *Pst*I site (-10 hexamers) and six different 28-mer/28-mer pairs with a 5' *Bam*HI site and a 3' *Sa*I site (-40 boxes). One *Bam*HI-*Sa*I module, one *Sa*I-*Pst*I module and the *Pst*I-*Hind*III module were ligated together in the vector pNM480 (Ap/Cb, ColE1 replicon, 8.6 kb; Minton, 1984), a translational *lacZ* fusion vector. The 3.2 kb *Bam*HI/*Aba*III fragment carrying the *lacZ* gene and various modules was taken from the pNM480 derivatives and cloned into the broad-host-range vector pKT240 (Bagdasarian *et al.*, 1983) cut with *Bam*HI and *Hpa*I, resulting in the plasmids pME3533, pME3534, pME3547, pME3548, pME3549, pME3769, pME3770, pME3771, pME3773, pME3780 and pME3781 (Ap/Cb *lacZ*⁺ Mob IncQ, 13.5 kb; Fig. 1, Tables 1-4). All promoter constructs were

verified by nucleotide sequencing. The plasmids were introduced into *P. aeruginosa* by electroporation as described by Zimmermann *et al.* (1991).

Enzyme assay. β -Galactosidase specific activities were measured in toluenized cells and are expressed in thousands of Miller units (kU) (Sambrook *et al.*, 1989). All enzyme assay data are mean values from at least three independent experiments. The measurements generally differed by < 20%. Arginine deiminase was assayed as described previously (Lüthi *et al.*, 1986).

RESULTS

Design of artificial modular promoters which depend on ANR/FNR activation

To study the functions of anaerobically inducible promoters in *P. aeruginosa* and *E. coli*, we constructed a series of synthetic promoters. Different -40 regions including the consensus FNR-binding site, the Arc box and various mutant derivatives were combined, via a *SaII* site, with either the *arc* -10 hexamer CCTAAT or the consensus -10 hexamer TATAAT of *E. coli* σ^{70} RNA polymerase (Fig. 1). These artificial promoters, which lack a typical -35 sequence, were inserted upstream of the *E. coli lacZ* gene with its own ribosome-binding site, in a broad-host-range IncQ vector plasmid. The prototype plasmid pME3533 (Fig. 1; Table 1) represents the wild-type *arc* promoter situation, except for the base changes introduced to create restriction sites (*Bam*HI, *Sa*II and *Pst*I) flanking the modules. In a previous study we have shown that the expression of the *arc* operon remains unchanged when the *arc* promoter region lying between positions -34 and -19 is substituted by a linker which is different from the *arc* wild-type sequence at every base, but without altering the spacing between the -40 and the -10 regions (Gamper *et al.*, 1991). Thus, the artificial *SaII* site (Fig. 1) is not likely to affect promoter activity.

Plasmid pME3781 (Fig. 1) contains the consensus FNR site and the consensus -10 hexamer. The different -40/-10 combinations are listed in Tables 1-4. A plasmid containing only the *lacZ* reporter gene, pME3536 (Fig. 1), gave negligible background β -galactosidase activities (Table 1) and may be useful as a promoter-probe system.

ANR, in contrast to FNR, recognizes the Arc box efficiently in *P. aeruginosa*

A first series of -40 regions was tested in combination with the *arc* -10 hexamer CCTAAT in *P. aeruginosa* as the host (Table 1). A strain having a deletion in the *anr* gene, PAO6261, gave low, virtually uninduced background values of β -galactosidase activity (Table 1). The ANR-positive wild-type strain PAO1 expressed high β -galactosidase levels which were inducible about 10-fold by oxygen limitation, when the -40 region corresponded to the consensus FNR site TTGAT....ATCAA or to the Arc box TTGAC....ATCAG (Table 1). This experiment confirms that ANR activates the artificial promoters used. Moreover, deletion of the first half-site of the Arc box (in pME3533-1) abolished induction, in agreement with

earlier results (Gamper *et al.*, 1991). The fact that the Arc box resulted in a β -galactosidase level that was about 30% below that specified by the FNR box (Table 1) was essentially due to the G at the 5th position of the second half-site rather than to the C at the 5th position of the first half-site (Table 1). The symmetrical -40 sequence CTGAT....ATCAG gave low, but detectable ANR-dependent induction (Table 1).

The *anr*-negative mutant PAO6261 (Ye *et al.*, 1995) does not grow anaerobically on nitrate nor does it utilize arginine as an anaerobic energy source via the arginine deiminase pathway. The *E. coli fnr* gene was introduced into this mutant on an IncP replicon (pRK2501*fnr*). FNR restored anaerobic growth of strain PAO6261 on nitrate but failed to induce the chromosomal *arc* operon: under oxygen-limiting conditions, arginine deiminase had a specific activity of 65 U mg⁻¹ in the wild-type PAO1 but remained at the uninduced level of 2.3 U mg⁻¹ in strain PAO6261/pRK2501*fnr*.

The promoter constructs were then tested in strain PAO6261 expressing FNR (Table 1). The consensus FNR site TTGAT....ATCAA allowed good induction by oxygen depletion. Although the induced β -galactosidase level was lower than that seen in the presence of ANR, it is clear that FNR of *E. coli* and RNA polymerase of *P. aeruginosa* do interact positively with this type of promoter. By contrast, when the promoter contained the Arc box TTGAC....ATCAG, activation by FNR was abolished almost totally (Table 1); this fact explains why FNR could not induce the chromosomal *arc* operon. FNR tolerated a T $\rightarrow$ C change at the fifth position of the first half-site in the FNR box but gave very poor induction when the 5th position of the second half-site was changed from A to G (Table 1). The symmetrical construct CTGAT....ATCAG was not recognized by FNR in *P. aeruginosa* (Table 1), corroborating earlier data obtained in *E. coli* (Lodge *et al.*, 1990). The results indicate that ANR and FNR differ with respect to recognition specificity, the base at the 5th position of the second half-site being especially important.

The consensus -10 hexamer TATAAT enhances aerobic and anaerobic expression in *P. aeruginosa*

The -40 regions described above were combined with the *E. coli* consensus -10 hexamer TATAAT instead of CCTAAT, and the expression of the *lacZ* reporter gene was again determined in *P. aeruginosa*. This change of the -10 sequence resulted in a three- to fourfold increase of both the aerobic and anaerobic expression levels and led to relatively high background activities in the *anr* mutant PAO6261 (Table 2). However, if we subtract these background activities from the values obtained in the presence of ANR (in strain PAO1) or FNR (in strain PAO6261/pRK2501*fnr*), then the differential activation abilities of ANR and FNR remain essentially unchanged. In particular, the Arc box allowed high expression with ANR under oxygen-limiting conditions whereas expression was much lower with FNR (Table 2). A control plasmid containing a very poorly conserved FNR

Table 2. Differential recognition of the –40 promoter region, combined with the consensus –10 hexamer TATAAT, by ANR and FNR in *P. aeruginosa*

Deviations from the consensus FNR box are indicated by bold letters. See first footnote of Table 1 for further details.

Plasmid	–40 Promoter region	β -Galactosidase specific activity (kU)					
		PAO1 (<i>anr</i> ⁺)		PAO6261/ pRK2501- <i>fnr</i> (<i>fnr</i> ⁺)		PAO6261 (<i>anr</i>)	
		+O ₂	–O ₂	+O ₂	–O ₂	+O ₂	–O ₂
pME3781	TTGAT....ATCAA	16.2	89.0	4.9	41.0	2.5	5.1
pME3534	TTGAC....ATCAG	11.2	75.0	2.0	15.6	1.9	3.8
pME3548	TTGAC....ATCAA	11.6	79.1	5.0	32.4	2.4	5.6
pME3769	TTGAT....ATCAG	11.0	80.9	2.6	14.7	1.4	3.5
pME3771	CTGAT....ATCAG	6.2	35.1	2.9	10.8	2.4	4.0
pME3771-1*	ACTGA....GACAG	5.2	8.9	7.7	8.7	6.8	5.9

* This construct is a spontaneous deletion derivative of pME3771: the T in the second half-site of the ANR box was deleted.

box (pME3771-1) showed no ANR- or FNR-dependent induction (Table 2), as expected.

In induced *P. aeruginosa* cells containing an ANR-responsive promoter (e.g. on pME3781), β -galactosidase represented at least 20 % of the total cellular protein (data not shown). This high expression was due, in part, to the fact that the IncQ vector used has about 30 copies in *P. aeruginosa* (Jeenes *et al.*, 1986). It could be argued that under these conditions the amount of ANR present in the cell might be sub-optimal and thus limit the expression. Therefore, we tested whether an increase of the *anr* copy number would enhance β -galactosidase expression. However, when the *anr* gene was introduced on the IncP plasmid pME3552 (Galimand *et al.*, 1991) into *P. aeruginosa*, the β -galactosidase levels specified by pME3781 did not change (data not shown).

In conclusion, the *E. coli* consensus –10 sequence TATAAT resulted in high aerobic and anaerobic expression in *P. aeruginosa*. In *E. coli* similar effects have been observed: the FNR-dependent *narGHJI* promoter shows high aerobic and anaerobic expression with a consensus –10 sequence (Walker & DeMoss 1991, 1992).

E. coli does not recognize the *P. aeruginosa* *arc* promoter

Two –40 regions (Arc box, FNR consensus) with the *arc* wild-type –10 hexamer and five –40 regions with the *E. coli* consensus –10 hexamer were also tested in *E. coli*. The effect of FNR was measured in strain MC1000 (*fnr*⁺) and that of ANR was assessed in strain JRG1728 (*fnr*⁺) carrying the *anr* gene in pME3777 (Table 3). The *fnr*-negative host JRG1728 gave negligible, uninduced β -galactosidase levels with all constructs tested (data not shown). From previous work (Galimand *et al.*, 1991; Sawers, 1991;

Zimmermann *et al.*, 1991) it is known that ANR can be expressed in *E. coli* and functionally replace FNR at some promoters. The native *anr* promoter is practically inactive in *E. coli*, giving $\leq 2\%$ of the expression found in *P. aeruginosa* (Sawers, 1991; Savioz *et al.*, 1993). Therefore, in pME3777 the *anr* gene was expressed from the *tac* promoter, which needed to be induced with IPTG in order to achieve complete activation of the anaerobically inducible promoters (data not shown).

The plasmid constructs containing the CCTAAT hexamer were expressed very poorly or not at all in *E. coli*; the slight FNR-dependent induction with the FNR consensus box was at the limit of detection in our routine β -galactosidase assays (Table 3). Exchanging CCTAAT with the consensus TATAAT led to high and regulated expression in *E. coli*. This indicates that the native *P. aeruginosa* –10 sequence constitutes a major barrier to expression of the *arc* operon in *E. coli*.

With TATAAT as the –10 hexamer, ANR activated promoters carrying the Arc box, the consensus FNR site or the single half-site mutant boxes (in pME3548 and pME3769) with similar efficiencies of 80–110 %, the anaerobic expression provided by the consensus FNR site being defined as 100 % (Table 3). These results are in good agreement with the parallel data obtained in *P. aeruginosa*, where the efficiencies of anaerobic expression varied between 85 and 100 % (Table 2). Recognition of the symmetrical CTGAT....ATCAG mutant –40 sequence by ANR appeared to be good in *E. coli* (80 % efficiency) and superior to that in *P. aeruginosa* (37 % efficiency; Tables 2 and 3), for reasons that are not obvious.

FNR effectively activated the promoters containing the consensus FNR site or the TTGAC half-site mutant

Table 3. Differential recognition of the –40 promoter region by ANR and FNR in *E. coli*

Deviations from the consensus FNR box are indicated by bold letters. See first footnote of Table 1 for further details.

Plasmid	–40 Region	–10 Region	β -Galactosidase specific activity (kU)			
			JRG1728/ pME3777 (<i>anr</i> ⁺)*		MC1000 (<i>fnr</i> ⁺)	
			+O ₂	–O ₂	+O ₂	–O ₂
pME3780	TTGAT....ATCAA	CCTAAT	< 0.01	< 0.01	0.01	0.04
pME3533	TTGAC....ATCAG	CCTAAT	< 0.01	< 0.01	< 0.01	< 0.01
pME3781	TTGAT....ATCAA	TATAAT	3.3	20.1	3.9	31.8
pME3534	TTGAC....ATCAG	TATAAT	1.7	17.3	2.4	13.1
pME3548	TTGAC....ATCAA	TATAAT	3.3	22.2	3.6	28.6
pME3769	TTGAT....ATCAG	TATAAT	1.5	16.3	2.0	14.4
pME3771	CTGAT....ATCAG	TATAAT	0.9	16.1	0.5	2.0

* The *fnr* mutant JRG1728 carries the *anr* gene of *P. aeruginosa* downstream of the *tac* promoter on pME3777 (see Methods). IPTG (1 mM) was used to induce the *tac* promoter.

Table 4. Differential recognition of –40 promoter regions, combined with the consensus –10 hexamer TATAAT, by ANR and FNR in *P. aeruginosa* and *E. coli*

Bold letters indicate the consensus FNR site as defined elsewhere in this study. The extended, symmetrical consensus FNR site of pME3773 has been proposed by Bell *et al.* (1989).

Plasmid	–40 Promoter region	Relative specific activity of LacZ reporter (%)*		
		ANR		FNR
		<i>P. aeruginosa</i> †	<i>E. coli</i> ‡	<i>E. coli</i> ‡
pME3781	GCTAT TTGAT GTGGATCAACATT	100	100	100
pME3773	AAAT-----A-AT-----AT--	40	66	98

* β -Galactosidase activities were determined in oxygen-limited cells (Tables 2 and 3) and set at 100%.

† The *Anr*⁺ hosts were the wild-type *P. aeruginosa* PAO1 and the *E. coli fnr* mutant JRG1728 carrying the *anr* gene of *P. aeruginosa* on pME3777.

‡ The *Fnr*⁺ host was *E. coli* MC1000.

(Table 3), as expected from previous mutant studies in *E. coli* (Jayaraman *et al.*, 1989; Bell *et al.*, 1990). The Arc box and the ATCAG half-site mutant gave reduced anaerobic expression in *E. coli* (efficiencies 41–44%; Table 3). This decrease was similar, albeit stronger, in *P. aeruginosa* (efficiencies 31–33%; Table 2). Finally, the symmetrical CTGAT....ATCAG sequence was poorly recognized by FNR in both *E. coli* and *P. aeruginosa*, confirming earlier results of Lodge *et al.* (1990) and Galimand *et al.* (1991). In conclusion, this symmetrical sequence reveals a major difference in recognition specificity between FNR and ANR. In general, the β -galactosidase levels measured in *E. coli* (Table 3) were lower than those in *P. aeruginosa* (Tables 1 and 2). This might be a consequence of the copy numbers of the IncQ vector used, estimated to be about

15 in *E. coli* and thus about half of that found in *P. aeruginosa* (Jeenes *et al.*, 1986).

A 22 bp extended consensus FNR site reduces ANR-dependent anaerobic activation

An extended, fully symmetrical consensus FNR site (Bell *et al.*, 1989) was constructed in plasmid pME3773 (Fig. 1) and compared to the FNR site used above in plasmid pME3781 where the TTGAT....ATCAA motif was flanked by nucleotides derived from the *arc* promoter (Table 4). In conjunction with the –10 sequence TATAAT, FNR activated both types of promoters similarly in *E. coli*. By contrast, ANR clearly preferred the FNR site flanked by the *arc* nucleotides over the fully

symmetrical FNR site, in both *E. coli* and *P. aeruginosa* (Table 4).

DISCUSSION

ANR and FNR can functionally replace each other under certain conditions

The cloned *anr* gene restores anaerobic growth to an *E. coli fnr* mutant on nitrate (Galimand *et al.*, 1991; Sawers, 1991) and, reciprocally, the cloned *fnr* gene complements a *P. aeruginosa anr* mutant for denitrification, as we have shown here. This implies that FNR and ANR can interact with the RNA polymerases of both *E. coli* and *P. aeruginosa*, provided that the promoters satisfy certain requirements; for instance, promoters which contain the consensus FNR site and the *E. coli* consensus -10 sequence meet these requirements. Neither *E. coli* (Sharrocks *et al.*, 1991; Walker & DeMoss, 1992; Sawers, 1993; Busby & Ebright, 1994) nor *P. aeruginosa* (Gamper *et al.*, 1991) requires a typical -35 promoter sequence for anaerobic induction by FNR/ANR. This fact does not rule out the possibility that the -35 region may influence the magnitude of FNR/ANR-dependent promoter activation.

The presumed loop around Gly-85 in FNR is essential for activating contacts between FNR and *E. coli* RNA polymerase (Bell & Busby, 1994). The 85-loop is strictly conserved in ANR (Spiro, 1994) and may thus facilitate contacts between ANR and RNA polymerase of *E. coli*. Furthermore, using the artificial FNR/ -10 consensus promoter we have shown here that FNR can activate RNA polymerase of *P. aeruginosa in vivo*. Other *E. coli* proteins that are known to activate *P. aeruginosa* RNA polymerase include RpoE (σ^E) and λ proteins cI and cII (Yu *et al.*, 1995; Gao & Gussin, 1991).

FNR could functionally complement an *anr* mutant of *P. aeruginosa* for denitrification. The chromosome region encoding nitrite reductase and nitric oxide reductase of *P. aeruginosa* contains several promoters that could be activated by ANR. For instance, the presumed ANR-binding sites in the *nirQ* and *norC* promoters are TTGAC.... ATCAA and TTGAT.... ATCAA, respectively (Arai *et al.*, 1994, 1995a). These sequences are well recognized by FNR in *P. aeruginosa* (Table 1). We expect that the control regions involved in the expression of other denitrification functions, e.g. nitrate reductase and nitrous oxide reductase, should also contain -40 regions that can interact with FNR, since the entire denitrification pathway of *P. aeruginosa* is anaerobically inducible (Ye *et al.*, 1995).

Some promoters carrying a mutant FNR-binding site are recognized more efficiently by ANR than by FNR

To facilitate the comparison of anaerobically inducible promoters in *P. aeruginosa* and *E. coli*, we used a set of modular constructs in a broad-host-range IncQ vector (Fig. 1). Because of the multi-copy nature of the vector, high β -galactosidase levels were observed in some cases, especially in *P. aeruginosa*. A chromosomal *arcD-lacZ* transcriptional fusion in *P. aeruginosa* PAO6265 gave an

<i>E. coli</i>	FNR 196	TRGDIGNYL GLT VETISRLLRFRQ	219
<i>P. aeruginosa</i>	ANR 191	SRNEIGNYL GLA VETVSRVFTFRQ	214
<i>P. stutzeri</i>	FnrA 191	SRNEIGNYL GLA VETVSRVFTFRQ	214

Fig. 2. Sequence alignment of the C-terminal helix-turn-helix motifs of FNR, ANR and FnrA. The FNR residues Glu-209, Ser-212 and Arg-213, which are known to have a significant role in the recognition of the FNR box, are conserved in the two *Pseudomonas* proteins. The residues shown in bold deviate between the *E. coli* and the two *Pseudomonas* proteins (Spiro, 1994).

uninduced (aerobic) value of 0.15 kU and an induced (anaerobic) value of 2.4 kU. Strain PAO1 carrying pME3533, which is a comparable multi-copy construct, had uninduced and induced β -galactosidase levels of 1.8 kU and 23.6 kU, respectively (values obtained after subtraction of the PAO6261 blanks; Table 1). Thus, the anaerobic induction factor is slightly higher in the mono-copy state than in the multi-copy situation. However, it is unlikely that in the latter situation expression was limited by the amount of the regulator.

The constructs containing the -10 hexamer TATAAT showed that ANR was a stronger activator than FNR at the -40 sequences TTGAC.... ATCAG (Arc box), TTGAT.... ATCAG, and CTGAT.... ATCAG in both *P. aeruginosa* and *E. coli* (Tables 2 and 3). The first two constructs show the same patterns of ANR- vs FNR-dependent expression in both hosts, within the accuracy of the method used. The construct carrying the -40 element CTGAT.... ATCAG was activated much better by ANR than by FNR in *E. coli* (Table 3). The same trend was observed in *P. aeruginosa*, but for unknown reasons the difference was less pronounced (Table 2). The structural basis for the differential promoter activation by ANR and FNR might be in the helix-turn-helix motifs of the regulators. The role of the FNR helix-turn-helix motif as a DNA-binding domain is well established (Spiro & Guest, 1987; Bell *et al.*, 1989; Lazazzera *et al.*, 1993; Spiro, 1994). ANR and the closely related FnrA protein of *P. stutzeri* (Cuypers & Zumft, 1993) have a helix-turn-helix motif which is very similar to that of FNR (Fig. 2). However, there are several FNR residues that are not conserved in ANR and FnrA (Fig. 2). Further work is needed to show whether these differences account for the differential activation specificities of FNR and ANR. Overall, our impression is that ANR may be less stringent in its DNA binding site specificity than FNR. Poor recognition of the *arc* promoter by FNR explains why FNR cannot complement an *anr* mutant of *P. aeruginosa* for induction of the arginine deiminase pathway.

RNA polymerases of *P. aeruginosa* and *E. coli* appear to have different -10 sequence specificities

The σ^{70} factors of *E. coli* and *P. aeruginosa* are very similar. Region 2 of σ^{70} , which is important for the recognition of -10 promoter regions, is 100% conserved in both micro-organisms (Tanaka & Takahashi, 1991; Ronald *et al.*, 1992). Somewhat unexpectedly, we found that the -10 *arc* hexamer CCTAAT gave different expression in *P.*

aeruginosa and *E. coli*. Good promoter activity in *P. aeruginosa* was contrasted by very low activity in *E. coli* (Tables 1 and 3). Among 300 *E. coli* promoters analysed, none has a CCTAAT –10 sequence (Lisser & Margalit, 1993). In *Pseudomonas*, a PyPuTAAAT consensus –10 sequence has been proposed (Ronald *et al.*, 1992; Py = C or T; Pu = A or G). Since this consensus sequence is based on a small number of promoters, its validity is uncertain. Our findings leave us with two interpretations. (i) In *P. aeruginosa*, transcription of anaerobically inducible promoters might involve an alternative σ factor. This possibility has not been ruled out but seems unlikely considering the excellent activity of promoters having a σ^{70} consensus –10 sequence in *P. aeruginosa*. (ii) It is more plausible to assume that in *P. aeruginosa*, by analogy with the situation in *E. coli* (Sharrocks *et al.*, 1991; Busby & Ebright, 1994), the ANR (FNR)-dependent promoters are transcribed by σ^{70} RNA polymerase. If such is the case, the –10 specificities of the σ^{70} RNA polymerases from the two organisms may be different. In support of this hypothesis, the *in vitro* data of Gao & Gussin (1991) suggest that purified σ^{70} RNA polymerase of *P. aeruginosa* may be less stringent than the *E. coli* enzyme in promoter recognition. In the context of the *arc* operon of *P. aeruginosa*, the –10 sequence CCTAAT allows the promoter to be tightly controlled by oxygen limitation. The consensus –10 sequence TATAAT leads to extremely high anaerobic expression, but also elevates the aerobic, uninduced expression.

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